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Selective Occlusion of Choroidal Neovascularization by Photodynamic Therapy With a Water-Soluble Photosensitizer, ATX-S10†

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Background and Objective: **To determine the optimal treatment parameters for selective occlusion of choroidal neovascularization (CNV) by photodynamic therapy (PDT) by using the photosensitizer ATX-S10 and a diode laser (wavelength = 670 nm).**
Materials and Methods: **Experimental CNV was induced in rat fundi by argon laser photocoagulation. The distribution of ATX-S10 in the chorioretina was analyzed by fluorescence microscopy, and the optimal treatment parameters for selective occlusion of CNV were investigated by changing the dosage and timing of laser irradiation. CNV closure and resulting damage of the surrounding tissue were documented by fluorescein angiography and light and electron microscopies.**
Results: **Fluorescence of ATX-S10 was observed to be localized in the vascular lumen of the retina and choroid within 5 min after dye injection and increased in intensity in CNV up to 2–6 h and decreased rapidly in normal tissue. Laser irradiation with radiant exposures of 7.4 J/cm^2 applied immediately after dye injection or with 22.0 J/cm^2 at 2–4 h later effectively occluded the induced CNV without causing significant damage to normal retinal capillaries and large choroidal vessels.**
Conclusions: **PDT using ATX-S10 can selectively occlude CNV. ATX-S10 is a potentially useful photosensitizer for the treatment of CNV. Lasers Surg. Med. 24:209–222, 1999.**

Key words: ATX-S10; choroidal neovascularization; photodynamic therapy; selective occlusion; treatment parameters

INTRODUCTION

Choroidal neovascularization (CNV) leads to severe visual loss in patients with age-related macular degeneration (AMD), the leading cause of blindness in the elderly. Laser photocoagulation is the only treatment shown to be somewhat effective in treating CNV [1–3]. However, laser photocoagulation acts not only on CNV but also the sensory retina because of heat emanating from the irradiation site, the retinal pigment epi-

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thelium. Such retinal damage is especially problematic in the treatment of subfoveal CNV [3] because severe visual loss is likely even if the CNV is successfully occluded. Therefore, occlusion of CNV that selectively spares surrounding normal tissue would be beneficial in the treatment of AMD. Photodynamic therapy (PDT), in which intravenous administration of a photosensitizer is followed by laser irradiation at the absorption peak of the photosensitizer, theoretically can destroy vascular endothelial cells [4] and induce thrombosis with subsequent regression of the new vessels, without any direct injury to surrounding normal tissue [5,6].

Many photosensitizers have been examined for the treatment of ocular neovascularization [7–20], but only benzoporphyrin derivative monoacid (BPD) [21] and tin ethyl etiopurpurin (SnET2) [22], which belong to the so-called class of second-generation photosensitizers, are presently under clinical trials and have been evaluated to be effective for treatment of this condition. Although hematoporphyrin derivatives (HPDs) have occlusive effects on ocular neovascularization and have been approved for clinical use in patients with certain kinds of cancer [7–9], they have the disadvantage of exhibiting long retention times in the body and correspondingly high residual phototoxicity to the skin. The ideal photosensitizer for use in occluding CNV would be highly photosensitive, relatively nontoxic both locally and systemically, and rapidly eliminated from the body, thereby facilitating shorter stays for the patient in a dark room after treatment. An additional requirement would be a long absorption wavelength because longer wavelength laser light has better transmission characteristics through ocular tissue, exudation, pigment, and hemorrhage that may be overlying CNV [24,25]. A new synthetic chlorine derivative, ATX-S10 (13,17-bispropionylaspartic acid-3-ethenyl-7-hydroxy-8-hydroxyiminoethylidene-2,7,12,18-tetramethyl-porphine; Toyo Hakka Kogyo Inc./ Laderly Japan Inc.; Fig. 1), has been developed with these criteria in mind [26]. The formula weight of this water-soluble dye is 927.79, which leads to favorable clearance rates from tissues. In previous investigations using rats and rabbits, we found that the concentration of ATX-S10 in the blood was halved within 30 min after administration and could not be detected at all after 24 h [27]. Also, ATX-S10 has an absorption peak at approximately 670 nm, which is advantageous in transmitting the sensitizing light to the treatment site. The absorption coefficient of ATX-S10 at 670 nm is 18,500 cm^{-1}. BPD and SnET2 also have these favorable absorption characteristics, but they also have the disadvantage of needing to be infused in the form of vehicles such as liposomes and emulsions because of their hydrophobicity. In contrast, the hydrophilicity of ATX-S10 enables a bolus intravenous injection and thus a reduced risk of thrombosis.

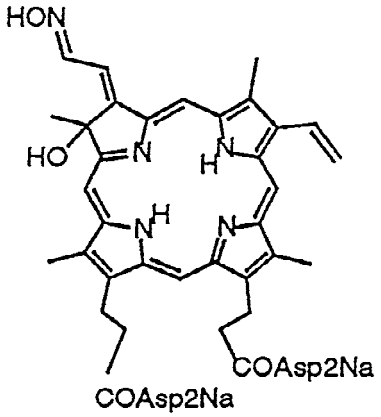

Fig. 1. The molecular structure of ATX-S10.

In the present study, we examined the distribution of ATX-S10 in the chorioretina by fluorescence microscopy at specified times after dye injection and investigated the optimal parameters for achieving selective occlusion of CNV, including the time of laser irradiation after dye injection and the effect of laser dosage.

MATERIALS AND METHODS

Animals

Twenty-six Long-Evans rats weighing 200–300 g were anesthetized for all procedures with intraperitoneal pentobarbital sodium (40 mg/kg body weight). Proparacaine HCl was used for topical anesthesia. Pupils were dilated with phenylephrine hydrochloride (2.5%) and tropicamide (0.8%). The treated eyes were enucleated for fluorescence microscopy and histologic examination under the same general and topical anesthesia, and animals were killed by injecting an overdose of sodium pentobarbital. The animals were at all times treated in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Induction of Experimental CNV

Experimental CNV was induced by irradiation with intense argon green laser (λ = 514 nm),

causing photocoagulation on the fundus, near the optic disc [28]. A 100-μm spot size was used for 0.1 sec duration and with a power of 140 mW. For the fluorescence microscopy study, a number of identical argon laser photocoagulations were performed in 24 eyes, and the eyes were examined on day 11 after photocoagulation, without preceding fluorescein angiography. For the PDT experiments, four CNV lesion sites were produced in each eye. On day 10, fundus photography and fluorescein angiography were performed to detect CNV. The eyes with confirmed CNV were treated by PDT on day 11.

Fluorescence Microscopy

The ATX-S10 was stored in a dark place before use and diluted with 0.9% saline just before injection. Sixteen milligrams per kilogram of ATX-S10 were injected into the tail vein in 12 rats with confirmed CNV. The eyes of the rats were enucleated at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 24 h after injection and were fixed with formaldehyde. Frozen sections of each eye were embedded in OCT compound (Tissue-Tek, Miles Scientific, Elkhar, IL) and prepared for viewing with a fluorescence microscope (BX50-34-FLAD1, Olympus, Tokyo, Japan) coupled with a cooled CCD camera and image processing system (ARGUS-50/C-CCD, Hamamatsu Photonics Inc., Hamamatsu, Japan) to pinpoint the location of the dye. The resulting 5-μ unstained frozen sections were first excited with 400–440-nm light, and the resulting emission light passed through a dichroic mirror (DM455, Olympus) and a 680-nm bandpass filter before detection with the CCD camera. The intensity and exposure time of excitation light were constant throughout these experiments. The intensity of fluorescence was measured with NIH imaging software (version 1.58). The ratio of fluorescence intensity of tissue to that of background was calculated. The normalized fluorescence intensity was graded on a scale of 1–5 according to the value of this ratio: –, ≤1; +, 1 <≤ 2.5; ++, 2.5 <≤ 5; +++, 5<≤ 10; ++++, 10 ≤. The specimens were also examined under a phase-contrast microscope. Frozen sections stained with hematoxylin and eosin were also observed by light microscopy to facilitate precise visual localization of the fluorescence.

As a control, four eyes of animals with confirmed CNV that had not been injected with dye were enucleated and similarly evaluated.

TABLE 1. Numbers of Lesions Investigated

Radiant exposure (J/cm^2)	Time (h) of irradiation after dye injection			
	0[a]	1	2	4
7.4	3	3	4	2
22.0	3	3	7	3
36.7	3	3	7	3
58.8	3	3	7	1

[a]Immediately after dye injection.

PDT

A diode laser (Hamamatsu Photonics Inc., Hamamatsu, Japan) emitting light at 670 nm was used as the irradiation source. The laser light was fed into a slit lamp (30 SL-M, Zeiss, Germany) through a 400-μm optical fiber and then delivered to the area of CNV through a cover glass in contact with the cornea. The dimmed light of the slit lamp was used only to focus the laser spot on the CNV and was turned off during PDT irradiation. The diameter of the laser beam on the retinal surface was approximately 500 μm, and the transmission rate of the 670-nm laser light through the ocular media was 60% ± 3, as measured by a power meter (Coherent Fieldmaster, Coherent, Auburn, CA) in preliminary experiments using three enucleated eyes. Irradiation was performed at four different times ranging from immediately after injection of ATX-S10 to up to 4 h later. Exposure time for PDT irradiance was set at 4 min. As had been found optimal for PDT from our preliminary experiment, the laser irradiances used were 0.1, 0.3, 0.5, and 0.8 mW/cm^2 at the corneal surface, which were calculated to be 30.6, 91.7, 152.9, and 244.9 mW/cm^2, respectively (or 7.4, 22.0, 36.7, and 58.8 J/cm^2, respectively), on the retinal surface. The number of lesions investigated for each treatment parameter set are presented in Table 1. As additional controls, two rats not injected with dye underwent identical PDT laser irradiation to both eyes, and one rat received dye administration without the subsequent laser irradiation.

Assessment of Therapeutic Selectivity

Fundus photographs taken with a fundus camera (Jenesis, Kowa, Tokyo, Japan) were taken at 1 h and 24 h after irradiation. After taking the photographs, fluorescein angiography was performed with 0.1 ml of 5% fluorescein sodium solution to document occlusion of the CNV, choriocapillaris, and retinal vessels. The eyes were enucleated 1 day after PDT and fixed in Karnovsky fixative (phosphate buffer, pH 7.4, 4°C).

TABLE 2. Time Course of the Intensity of ATX-S10 Fluorescence in the Rat Fundus After Dye Injection*

	Time after ATX-S10 injection						
Tissue	5 min	30 min	1 h	2 h	4 h	6 h	24 h
Retina							
RC lumen	+	+	–	–	–	–	–
RV lumen	++	+	+	–	–	–	–
RA wall	+++	++	++	+	+	–	–
Outer segments	+	++	+	+	–	–	–
RPE or Bruch's membrane	++	+++	+	+	+	+	–
CNV	++	+++	+++	+++	++	++	–
Choroid							
CC lumen	++	++	+	–	–	–	–
CV lumen	++++	+++	++	+	–	–	–
CA wall	++++	++++	++++	+++	+++	++	–
Sclera	++++	++++	++++	++++	++++	++	–

*Fluorescence intensity was analyzed under a fluorescence microscope and graded according to the ratio of fluorescein intensity of the tissue to that of background: –, ≤1; +, 1 < ≤2.5; ++, 2.5 < ≤5; +++, 5 < ≤10; ++++, 10 ≤. RC, retinal capillary; RV, retinal artery and vein; RA, retinal artery; RPE, retinal pigment epithelium; CNV, choroidal neovascularization; CC, choriocapillaris; CV, choroidal artery and vein; CA, choroidal artery.

Treated lesions were dissected out and placed in the fixative overnight. Tissue samples were postfixed in 2% osmium tetroxide (4°C), dehydrated in serial ethanol, and embedded in polybed. Thick sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with electron microscopy (JEM-1200EX, Japan Electron Optics Laboratory, Tokyo, Japan).

Therapeutic selectivity was evaluated in terms of the extent of damage to the CNV, retinal capillaries, and large choroidal vessels in the treated area based on fluorescein angiographic and histologic observation at 1 day after PDT. In cases in which the CNV was completely occluded but the retinal capillaries and large choroidal vessels were not, the selectivity of the treatment was rated as good. When the CNV, retinal capillaries, and large choroidal vessels were all occluded, selectivity was rated as poor.

RESULTS

Fluorescence Microscopic Findings

The results of the fluorescence microscopy study are summarized in Table 2. At 5 min after dye injection, fluorescence of ATX-S10 appeared in the CNV. Fluorescence intensity increased, reaching a peak at 30 min, 1 h (Fig. 2), and 2 h (Fig. 3) and decreased, weakly persisting at 6 h.

In the choroid, there was observable fluorescence in the lumen of the choriocapillaris and in the choroidal arteries and veins, and the choroidal artery wall was heavily stained with ATX-S10 from 5 min to 1 h after injection (Fig. 4). At 2 h after injection, fluorescence in the lumen of the large choroidal vessels had diminished, and no fluorescence was observed in the choriocapillaris.

In the retina, there was prominent fluorescence from the arterial wall and weak fluorescence in the lumen of the retinal capillaries at 5–30 min after injection (Fig. 5). At 30 min after injection, fluorescence from the retinal outer segments and from the basal side of the retinal pigment epithelium was observed; the fluorescence decreased 1 h after injection. The fluorescence from the basal side of retinal pigment epithelium was thought to represent selective staining of Bruch's membrane.

The sclera showed prominent fluorescence from 5 min to 4 h after injection. By 24 h after injection, the fluorescence had subsided in all tissues. No comparable fluorescence was detected in any tissue from the control animals.

Fundus Photographic and Fluorescein Angiographic Results

There were no apparent color changes in any treated areas immediately after PDT. The findings from fundus photographs taken 1 h after PDT are summarized in Table 3. The lesions treated immediately or 1 h after dye injection showed grayish color change in the deep retina, whereas most of the lesions treated 2 or 4 h after dye injection showed no color changes at any level of exposure. A narrowing of retinal arteries and veins irradiated immediately after injection was observed in the lesions irradiated with 22.0 J/cm^2 and higher.

At 1 day after PDT, different extents of deep

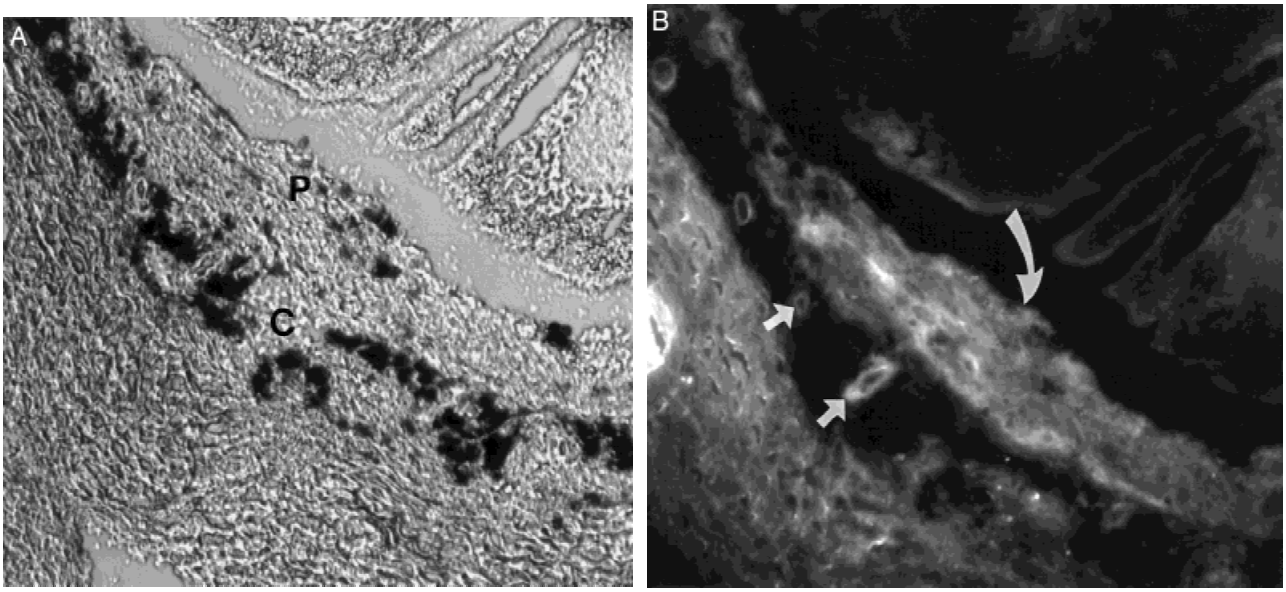

Fig. 2. Choroidal neovascular tissue 1 h after administration of ATX-S10 (16 mg/kg). **A:** Phase-contrast micrograph. C, choroid; P, subretinal proliferative tissue. **B:** Fluorescence micrograph of the same section shows fluorescence from subretinal proliferative tissue (curved arrow). Fluorescence from the choroidal vessel walls (arrows) and sclera are also noted. Original magnification, ×20.

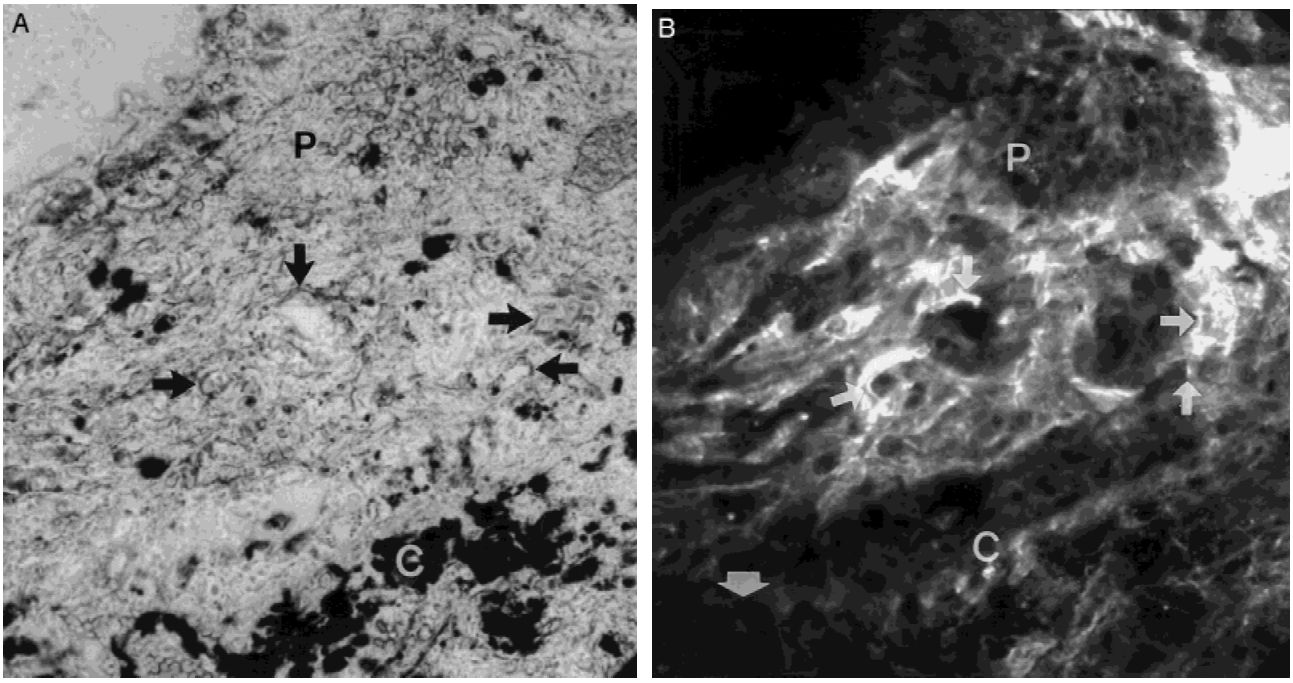

Fig. 3. Choroidal neovascular tissue 2 h after administration of ATX-S10 (16 mg/kg). **A:** Phase-contrast micrograph. Arrows indicate new vessels. **B:** Fluorescence micrograph of the same section shows prominent fluorescence from whole subretinal proliferative tissue (P). Bright fluorescence is also noted from the new vessel wall (arrows). The large choroidal vein (thick arrow) shows no fluorescence. C, choroid. Original magnification, ×40.

retinal whitening appeared, depending on the intensity of irradiation (Table 3, Fig. 6). The area of whitening usually corresponded to the size of the laser irradiation spot, but the whitish area of the lesions treated at an exposure of 58.8 J/cm^2 immediately after dye injection was much larger than the laser irradiation spot. Table 4 summarizes the fluorescein angiographic findings 1 day

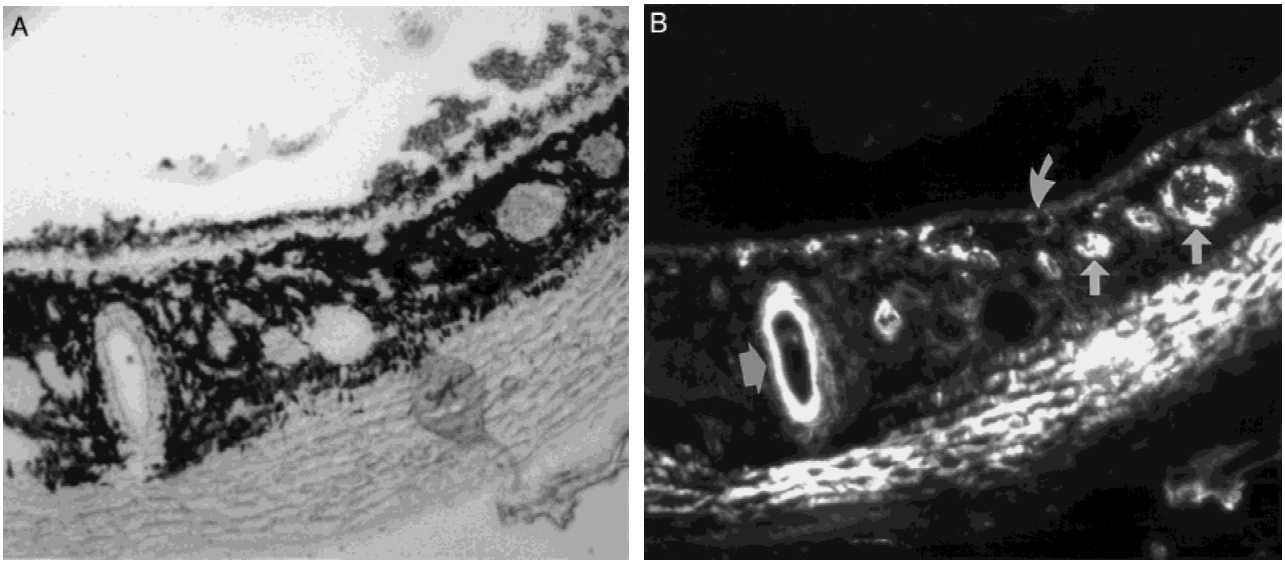

Fig. 4. The choroid 5 min after administration of ATX-S10 (16 mg/kg). **A:** Phase-contrast micrograph. **B:** Fluorescence micrograph of the same section shows prominent fluorescence from the walls of choroidal artery (thick arrow) and the lumen of large choroidal vessels (small arrows). Fluorescence from the choriocapillaris (curved arrow) and weak fluorescence at the basal side of retinal pigment epithelium are visible. Original magnification, ×20.

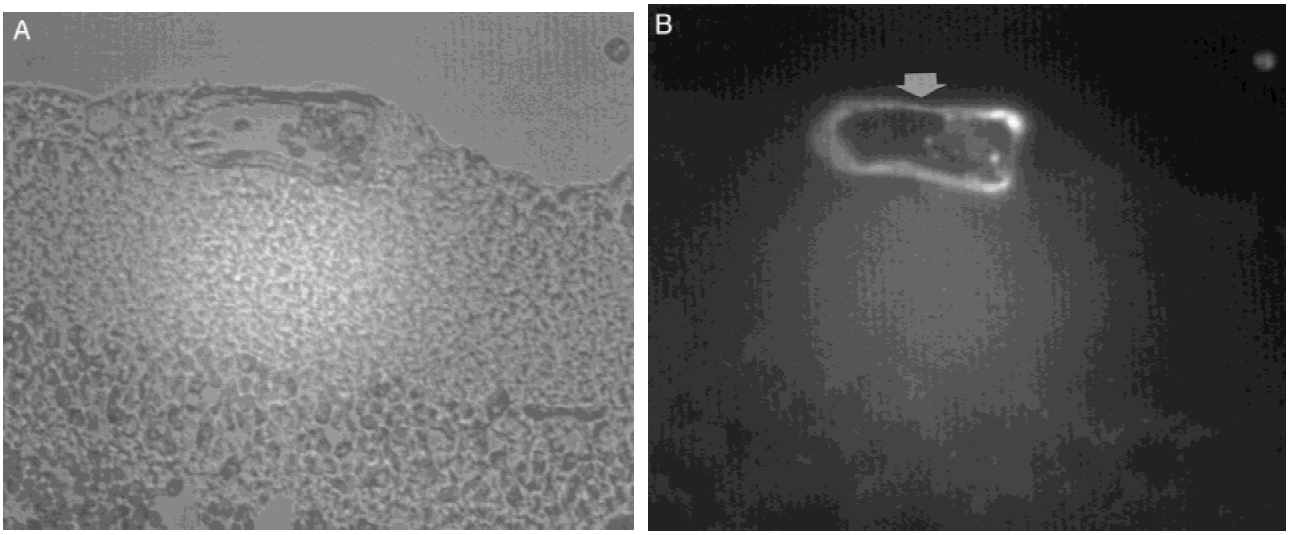

Fig. 5. The retina 5 min after administration of ATX-S10 (16 mg/kg). **A:** Phase-contrast micrograph. **B:** Fluorescence micrograph of the same section shows prominent fluorescence from the retinal artery (arrow). Original magnification, ×20.

after PDT. The degree of angiographic closure of CNV, choriocapillaris, retinal capillaries, and retinal arteries and veins depended on the dose of irradiation and the time interval between dye injection and irradiation. The fluorescein angiography showed that CNV closure was achieved without complete closure of the retinal capillaries when PDT was performed immediately after dye injection with irradiation at 7.4 J/cm^2 and at 2 or 4 h after dye injection with irradiation at 22.0 J/cm^2 (Fig. 6). The retinal and choroidal vessels, including CNV, were not occluded in the corresponding control eyes.

Histologic Findings

Light microscopic findings at 1 day after PDT are summarized in Table 5. In the lesions irradiated immediately after dye injection with low intensity (7.4 J/cm^2), the CNV and choriocapillaris were occluded by thrombus, whereas most of the retinal capillaries and large choroidal ves-

TABLE 3. Ophthalmoscopic Findings of Treated Lesions After Photodynamic Therapy*

Radiance (J/cm^2)	Time (h) of irradiation after dye injection			
	0[a]	1	2	4
		After 1 h		
7.4	+	+	–	–
	(0/3)	(0/3)	(0/3)	(0/3)
22.0	+	+	–	–
	(2/3)	(2/3)	(0/3)	(0/3)
36.7	+	+	–~+	–~+
	(2/3)	(0/3)	(0/3)	(0/3)
58.8	+	+	–	nt
	(2/3)	(0/3)	(0/3)	
		After 1 day		
7.4	+	+~++	–	–
	(0/3)	(2/3)	(0/3)	(0/3)
22.0	+~++	++	+~++	+~++
	(2/3)	(2/3)	(0/3)	(0/3)
36.7	++	++	+~++	++
	(2/3)	(2/3)	(1/5)	(1/2)
58.8	++~+++	++~+++	++	++
	(2/3)	(2/3)	(3/5)	(0/3)

*Color changes were identified by fundus photography: –, no change; +, grayish; ++, whitish; +++, densely whitish. The number in the parenthesis indicates the number of lesions representing the narrowing of retinal arterioles and venules per total number of lesions examined.
[a]Immediately after dye injection.

sels were not closed. Thus, selectivity was achieved with these treatment parameters. In contrast, the retinal capillaries, CNV, choriocapillaris, and most of the large choroidal vessels were occluded by thrombus in the lesions irradiated immediately after dye injection with irradiation intensities of 22.0 J/cm^2 and higher.

In the lesions treated 1 h after dye injection, the retinal capillaries, CNV, choriocapillaris, and large choroidal vessels were occluded at all laser radiances. Thus, no selective occlusion of the CNV was achieved at this time point.

In the lesions treated 2 or 4 h after dye injection, laser radiance of 7.4 J/cm^2 seemed insufficient to occlude the CNV. With irradiation of 22.0 J/cm^2, the CNV was occluded without occlusion of the retinal capillaries and large choroidal vessels (Fig. 7). A small number of retinal arteries were shrunken, and thrombus formation was observed on the inner surface of the arteries, but most of the retinal arteries and veins appeared intact. Thus, at these times, selective occlusion of the CNV was achieved with these treatment parameters. No retinal and choroidal vessels, including CNV, were occluded in the corresponding control eyes.

At electron microscopic examination of the lesions in which selective occlusion of the CNV was achieved, the endothelium of the CNV appeared in some parts as thin, electron-dense cytoplasm and was disrupted in others (Fig. 8). Most of the pericytes were well preserved, and the basement membrane of the CNV appeared intact. The proliferative retinal pigment epithelium and fibrous cells surrounding the CNV were not damaged. The endothelium of the choroiocapillaris was also disrupted. The endothelium of the large choroidal vessels showed no damage of cellular organelles, although platelet adhesion was observed on the surface of the endothelium in some lesions. Damage of the sensory retina accompanying laser photocoagulation for CNV induction was observed in all of the lesions investigated, but there was no apparent difference in damage between the PDT lesions and the control lesions. No apparent changes were evident in the ganglion cells and neurofibers. The sclera showed the normal arrangement of collagen fibers.

DISCUSSION

Because the irradiation of 30.6–244.9 mW/cm^2 used in the present study is too low to induce thermal coagulation [19], it must be concluded that the treatment effects observed were the result of photodynamic rather than thermal action. Consistent with this conclusion was the delayed retinal whitening of the treated lesions, secondary to damage of parts of the vascular system such as the choriocapillaris. In the present study,

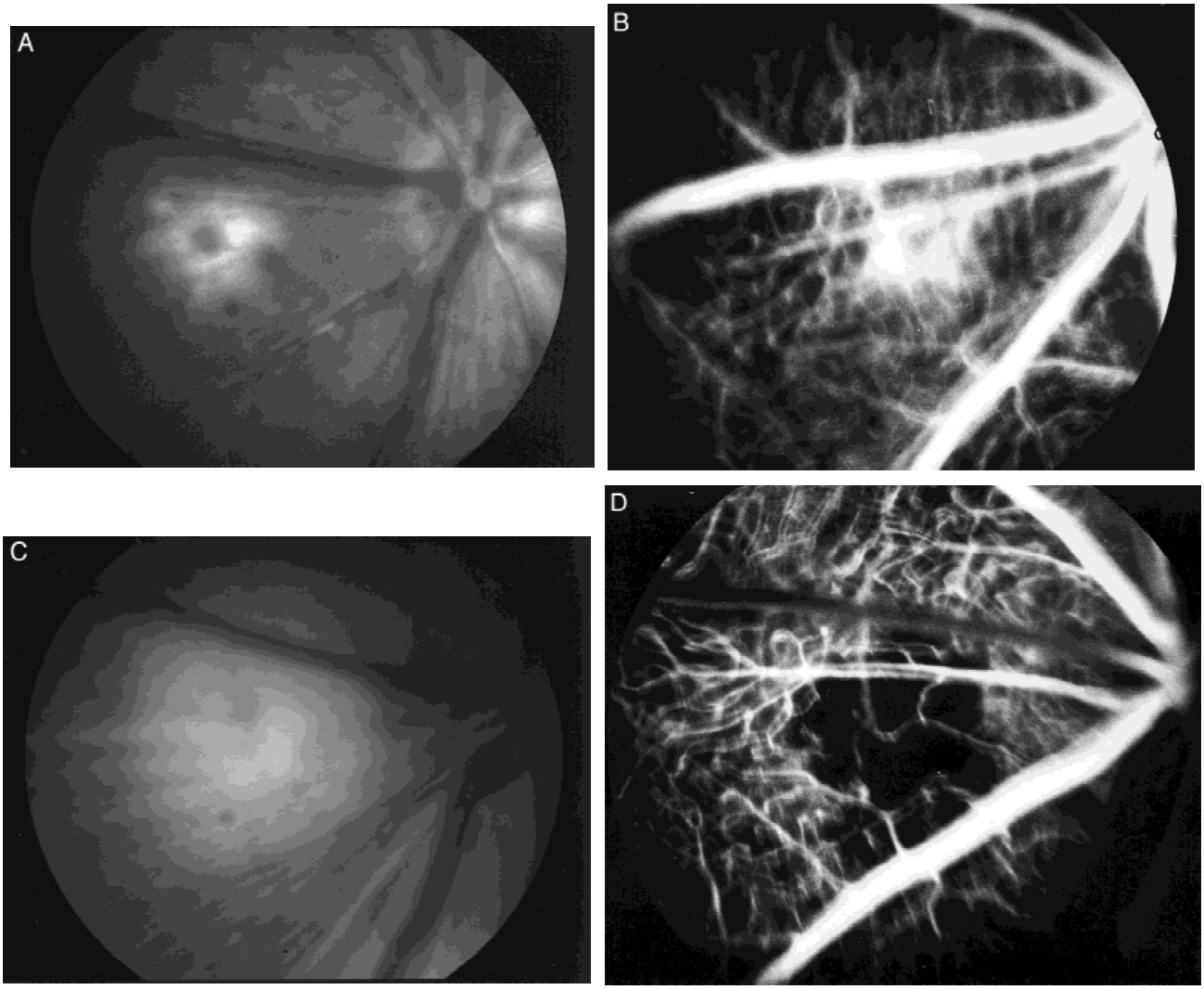

Fig. 6. Photodynamic therapy (PDT)-induced closure of choroidal neovascularization (CNV) using ATX-S10. **A:** Fundus photograph of CNV taken before PDT. Note the whitish laser burn. **B:** Fluorescein angiogram of CNV taken before PDT. Hyperfluorescence with fluorescein leakage is noted in the area of the CNV. **C:** Fundus photograph of CNV 1 day after PDT, using 16 mg/kg of ATX-S10 at an irradiation dose of 22 J/cm^2. There is mild whitening of the deep retina in the treated area. **D:** Early-phase fluorescein angiogram of the fundus. The lesion shows hypofluorescence indicative of occlusion of CNV, with strong perfusion of overlying retinal capillaries.

we tested different irradiation timings and light doses under a fixed dose of ATX-S10 to determine which parameters resulted in selective closure of CNV.

We found that the optimal parameters for selective occlusion of the experimentally induced CNV with 16 mg/kg of ATX-S10 were a light dose of 7.4 J/cm^2 administrated immediately after dye injection and a dose of 22 J/cm^2 administrated at 2–4 h after dye injection. With these treatment parameters, the CNV was occluded with thrombus, whereas damage to retinal and large choroidal vessels remained minimal. It was notable that selectivity was poor with PDT performed at 1 h after dye injection and that the optimal timing of laser irradiation proved to be either immediately after dye injection, or a few hours following dye injection. These irradiation time periods are consistent with findings in an investigation of corneal neovascularization [29]. In that study, selective occlusion of corneal neovascularization was achieved by PDT performed both immediately and 4 h after dye injection. Different optimal time periods for laser irradiation have been reported for other dyes. For PDT using chloro-aluminum sulfonated phthalocyanine, the optimal time was immediately after dye injection [14]. For BPD, the optimal was suggested to be 1–2 h after dye injec-

TABLE 4. Fluorescein Angiographic Evaluation of the Degree of Vascular Occlusion 1 Day After Photodynamic Therapy*

Radiance (J/cm^2)	Tissue	Vessels	Time (h) of irradiation after dye injection			
			0[a]	1	2	4
7.4	Retina	Capillaries	+/–	+	–	–
		Large vessels	–	NL	–	–
	Choroid	New vessels	+	+	–	–
		Capillaries	+/–	+	–	–
22.0	Retina	Capillaries	+	+	+/–	+/–
		Large vessels	NL	NL	–	–
	Choroid	New vessels	+	+	+	+
		Capillaries	+	+	+/–	+/–
36.7	Retina	Capillaries	+	+	+	+
		Large vessels	NL	NL	–	L
	Choroid	New vessels	+	+	+	+
		Capillaries	+	+	+	+
58.8	Retina	Capillaries	+	+	+	+
		Large vessels	NL	NL	L	–
	Choroid	New vessels	+	+	+	+
		Capillaries	+	+	+	+

*–, No change; +/–, partly closed; +, closed; L, leakage only; NL, narrowing with leakage.
[a]Immediately after dye injection.

TABLE 5. Light Microscopic Evaluation of the Degree of Vascular Occlusion 1 Day After Photodynamic Therapy*

Radiance (J/cm^2)	Tissue	Vessels	Time (h) of irradiation after dye injection			
			0[a]	1	2	4
7.4	Retina	Capillaries	+/–	+	–	–
	Choroid	New vessels	+	+	+/–	+/–
		Capillaries	+	+	–	–
		Large vessels	+/–	+	–	–
22.0	Retina	Capillaries	+	+	–	–
	Choroid	New vessels	+	+	+	+
		Capillaries	+	+	+	+
		Large vessels	+/–	+	–	–
36.7	Retina	Capillaries	+	+	+	+
	Choroid	New vessels	+	+	+	+
		Capillaries	+	+	+	+
		Large vessels	+	+	–	–
58.8	Retina	Capillaries	+	+	+	+
	Choroid	New vessels	+	+	+	+
		Capillaries	+	+	–	–
		Large vessels	+	+	–	–

*–, open; +/–, partly closed; +, closed.
[a]Immediately after dye injection.

tion in an investigation of corneal neovascularization [18] and 20–50 min in other CNV investigations [19,20]. With PDT using HPDs, the optimal timing suggested is usually 2 or 3 days after dye injection [9].

We concluded from the present fluorescence microscopy studies that ATX-S10 uptake by the CNV increased during the initial 30 min after dye injection and persisted for up to 6 h. By contrast, ATX-S10 was taken up by retinal and choroidal vessels immediately after dye injection and decreased after 1 h after injection. This accumulation pattern was consistent with that reported by previous investigations of corneal neovascularization, which demonstrated semiquantitatively the amount of ATX-S10 uptake in vivo when using pulsed nitrogen laser ($\lambda = 337$ nm) fluorospectrometry [30]. Therefore, it is clear that, because the highest level of dye accumulation in both the CNV and normal vessel wall occurs within 1 h after dye injection, PDT at this time cannot produce selective occlusion of the CNV. The best time

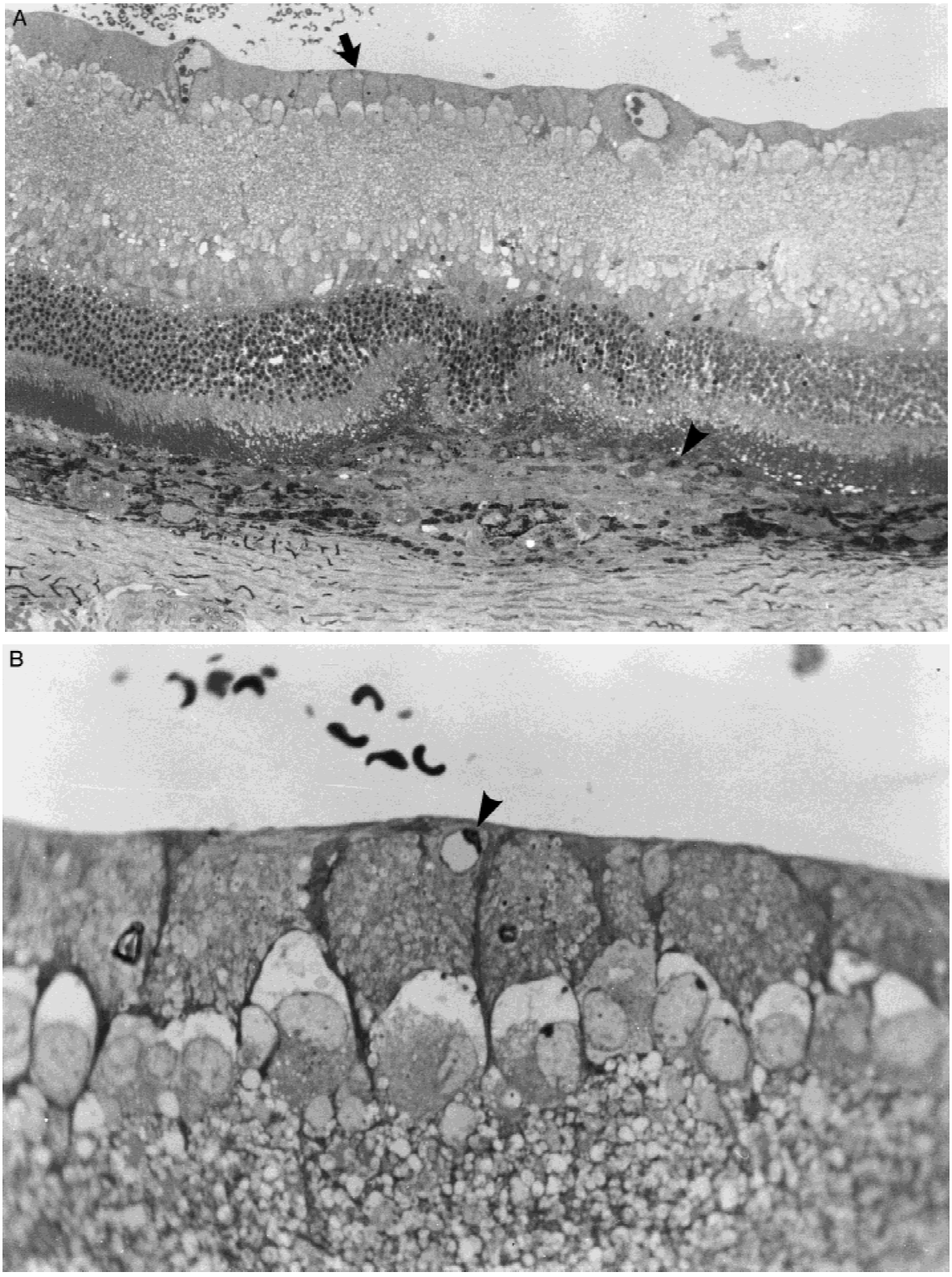

Figure 7

presumably would be 2–4 h after dye injection, when the difference between the amount of ATX-S10 in the CNV and that in the retinal and choroidal vessel walls is greatest. It is unlikely that the different amounts of the dye in the CNV versus that in the retinal and choroidal vessels ac-

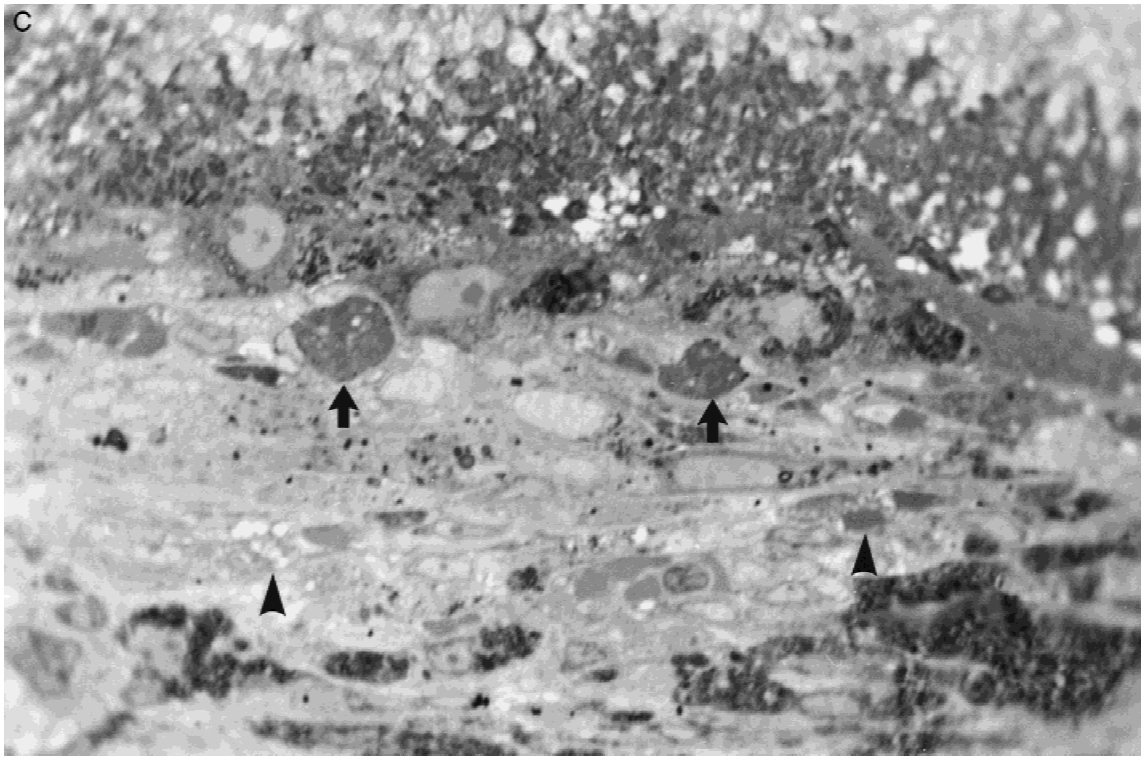

Fig. 7. (Continued) Light micrographs taken 1 day after photodynamic therapy with 22.0 J/cm^2 of a lesion treated 2 h after dye injection. **A:** Outer nuclear layer cells and photoreceptors were damaged by the laser photocoagulation for induction of choroidal neovascularization, whereas the ganglion cell layer and neurofiber layer were well preserved. Toluidine blue stain. Original magnification, ×50. **B:** High magnification of the region indicated by the arrow in A. The retinal capillary was open (arrowhead). Original magnification, ×250. **C:** High magnification of the region indicated by the arrowhead in A. The choroidal new vessels (arrows) and choriocapillaris (arrowheads) were closed with thrombus. Original magnification, ×250.

count for the selective occlusion achieved by PDT performed immediately after injection because at this point, as shown by fluorescence microscopy, most of the dye is still in the plasma. Rather, we suggest that, because in the present experimental model fluorescein angiography showed that the network pattern of the CNV appeared a little later than the choroidal and retinal filling, the circulation time may be slower in the CNV than in the retinal capillaries and choroidal vessels. Classic CNV usually fluoresces during the choroidal filling stage, but delayed filling may occur in some cases, depending on pathologic status [31,32]. The relatively slow blood flow through the CNV may increase photodynamic effects or thrombus formation. Another possibility is that the exact mechanism of photodynamic injury in the CNV may differ from that in normal retinal and choroidal vessels.

The localization of ATX-S10 we observed in the present study differs from that reported for HPDs and lipoprotein-delivered BPD [33,34]. HPDs and BPD, which are highly lipophilic, accumulate in the retinal pigment epithelium immediately after dye administration and do not accumulate in the sclera. By contrast, ATX-S10, which is a water-soluble dye, accumulates in the sclera immediately after dye administration. These differences are likely due to differences in lipophilicity and other characteristics such as molecular weight, charge distribution, etc. [34]. In the present study, no histologic damage was observed in sclera with any of the PDT treatment parameters, despite the high affinity of ATX-S10 for scleral tissue.

In the present study, even using treatment parameters that achieved selective occlusion of the CNV, the choriocapillaris was not spared, and retinal and choroidal vessels were damaged, even when the PDT laser dose was moderately low because normal tissue also uptakes some ATX-S10. Therefore, even with optimal parameters, focal irradiation would be essential for clinical treatment. It is important in this regard to develop optimal delivery equipment that allows for precise irradiation of a target lesion.

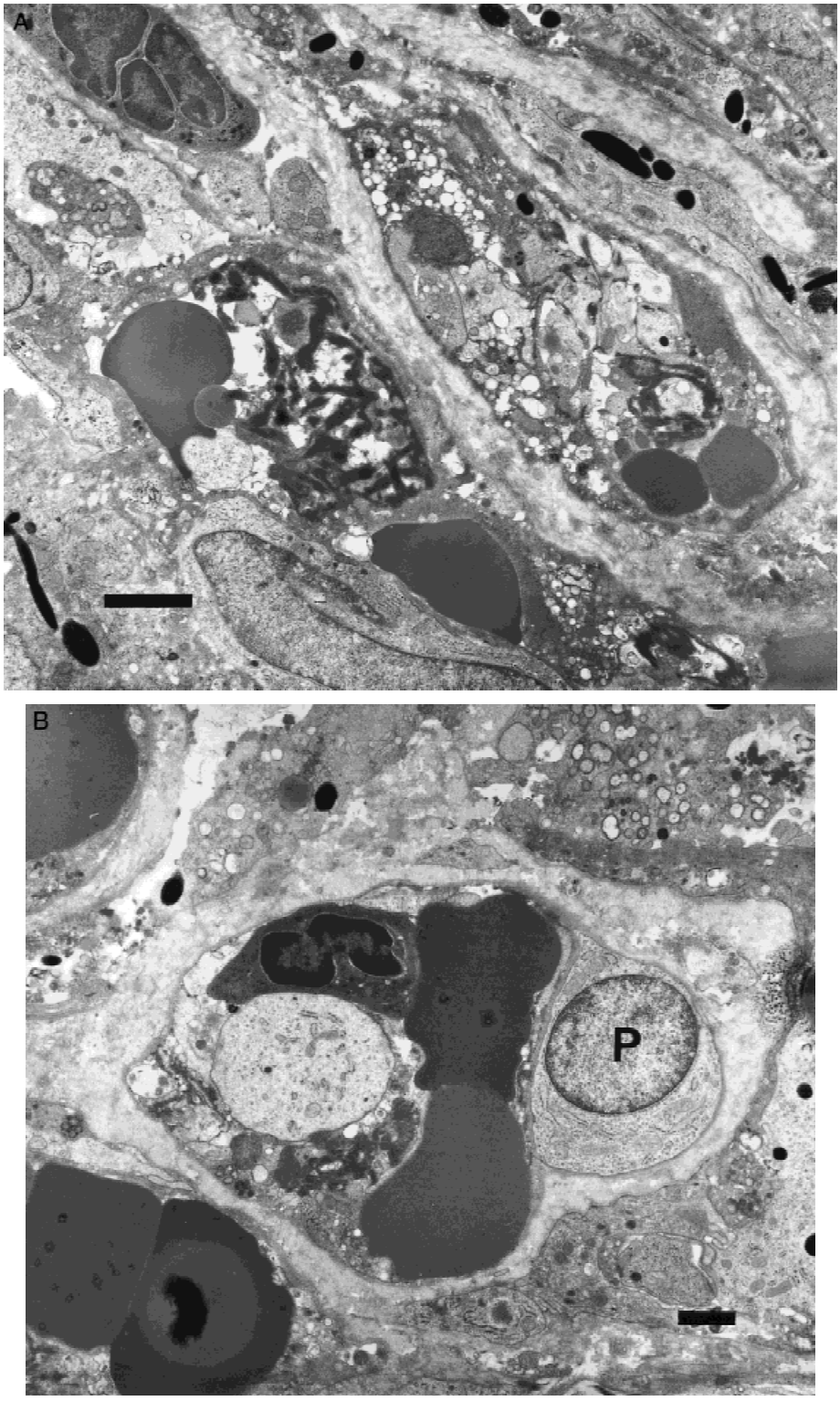

Fig. 8. Electron micrographs taken 1 day after photodynamic therapy with 22.0 J/cm^2 of a lesion treated 2 h after dye injection. **A:** The choroidal new vessel is filled with platelets, erythrocytes, and fibrin. Endothelial cells have been stripped. Surviving metaplastic retinal pigment epithelial cells are seen adjacent to a new vessel. **B:** The endothelial cells of the new vessel are disrupted, but the basement membrane and pericytes (P) were preserved. Scale bars = 2 μm in A, 1 μm in B.

We have shown the effectiveness of PDT when using ATX-S10 for the treatment of CNV. We used a fixed dye dose, which was chosen for the rat from our experiences in previous studies and from preliminary investigations. Kramer et al. [20] suggested that reduction of the dye dosage can increase treatment selectivity. As a next step, we intend to investigate optimal treatment parameters with different dye doses. This is now under investigation in a primate model. In the present study, damage to the retina was evaluated from assessing the closure of the retinal capillaries, but some retinal capillaries had been already occluded by the laser photocoagulation used to induce CNV before PDT treatment. Therefore, the evaluation of the retinal damage by PDT with the optimal treatment parameters obtained in the present study should be rechecked in the normal retina. Also, a preliminary clinical trial using BPD has shown that recurrence can be a problem [22]. The persistence of pericytes in new vessel walls observed in the present study suggests that neovascularization may easily recur. Therefore, it also will be necessary to investigate the long-term efficacies of PDT using ATX-S10 as the photosensitizer.

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REFERENCES

1. Macular Photocoagulation Study Group. Argon laser photocoagulation for neovascular maculopathy. Three-year results from randomized clinical trials. Arch Ophthalmol 1986;104:694–701.
2. Macular Photocoagulation Study Group. Krypton laser photocoagulation for neovascular lesions of age-related macular degeneration. Results of a randomized clinical trial. Arch Ophthalmol 1990;108:816–824.
3. Macular Photocoagulation Study Group. Laser photocoagulation of subfoveal neovascular lesions of age-related macular degeneration. Updated findings from two clinical trials. Arch Ophthalmol 1993;111:1200–1209.
4. Berenbaum MC, Hall GW, Hoyes AD. Cerebral photosensitization by haematoporphyrin derivative. Evidence for an endothelial site of action. Br J Cancer 1986;53:81–89.
5. Nelson JS, Liaw LH, Oresnstein A, Roberts WG, Berns MW. Mechanism of tumor destruction following photodynamic therapy with hematoporphyrin derivative, chlorine, and phthalocyanine. J Natl Cancer Inst 1988;80: 1599–1605.
6. Fingar VH, Wieman TJ, Wiehle SA, Cerrito PB. The role of microvascular damage in photodynamic therapy: the effect of treatment on vessel constriction, permeability, and leukocyte adhesion. Cancer Res 1992;52:4919–4921.
7. Packer AJ, Tse DT, Gu X-Q, Hayreh SS. Hematoporphyrin photoradiation therapy for iris neovascularization. A preliminary report. Arch Ophthalmol 1984;102:1193–1197.
8. Thomas EL, Langhofer M. Closure of experimental subretinal neovascular vessels with DHE augmented argon green laser photocoagulation. Photochem Photobiol 1987; 46:881–886.
9. Obana A, Gohto Y, Matsumoto M, Miki T. Photodynamic therapy of corneal neovascularization using hematoporphyrin derivatives. J Jpn Soc Laser Med 1996;17:31–43.
10. Huang AJN, Watson BD, Hernandez ZE, Tseng SCG. Photothrombosis of corneal neovascularization by intravenous rose bengal and argon laser irradiation. Arch Ophthalmol 1989;106:680–685.
11. Corrent G, Roussel TJ, Tseng SCG, Watson BD. Promotion of graft survival by photothrombotic occlusion of corneal neovascularization. Arch Ophthalmol 1989;107: 1501–1506.
12. Epstein RJ, Hendricks RL, Harris DM. Photodynamic terapy for corneal neovascularization. Cornea 1991;10: 424–432.
13. Pallikaris IG, Tslimbaris MK, Iliaki OE, Naoumidi II, Georgiades A, Panagopoulos IA. Effectivness of corneal neovascularization photothrombosis using phthalocyanine and a diode laser (675nm). Laser Surg Med 1993; 13:197–203.
14. Tsilimbaris MK, Pallikaris IG, Naoumidi II, Vlahonikolis IG, Tsakalof AK, Lydataki SE. Phthalocyanine mediated photodynamic thrombosis of experimental corneal neovascularization: effect of phthalocyanine dose and irradiation onset time on vascular occlusion rate. Laser Surg Med 1994;15:19–31.
15. Miller JW, Stinson WG, Gregory WA, El-Koumy HA, Puliafito CA. Phthalocyanine photodynamic therapy of experimental iris neovascularization. Ophthalmology 1991; 98:1711–1719.
16. Kliman GH, Puliafito CA, Stern D, Borirakchanyavat S, Gregory WA. Phthalocynanine photodynamic therapy: new strategy for closure of choroidal neovascularization. Laser Surg Med 1994;15:2–10.
17. Schmidt-Erfurth U, Hasan T, Gragoudas E, Michaud N, Flotte TJ, Birngruber R. Vascular targeting in photodynamic occlusion of subretinal vessels. Ophthalmology 1994;101:1953–1961.
18. Schmidt-Erfurth U, Hasan T, Schomacker K, Flotte TJ, Birngruber R. In vivo uptake of liposomal benzoporphyrin derivative and photothrombosis in experimental corneal neovascularization. Laser Surg Med 1995;17:178–188.
19. Miller JW, Walsh AW, Kramer M, et al. Photodynamic therapy of experimental choroidal neovascularization using lipoprotein-derived benzoporphyrin. Arch Ophthalmol 1995;113:810–818.
20. Kramer M, Miller JW, Michaud N, et al. Liposomal benzoporphyrin derivative verteporfin photodynamic therapy. Ophthalmology 1996;103:427–438.
21. Gragoudas ES, Schmidt-Erfurth U, Sickenberg M, et al. Results and preliminary dosimetry of photodynamic

therapy for choroidal neovascularization in age-related macular degeneration in a phase I/II study. Invest Ophthalmol Vis Sci 1997;38:S17.
22. Thomas EL, Rosen R, Murphy R, et al. Purlytin™ (SnET2)-photodynamic therapy produces closure of subfoveal choroidal neovascularization in humans. Invest Ophthalmol Vis Sci 1998;39:S242.
23. Obana A, Miki T. Photocoagulation through retinal edema using three different wavelength lasers. Lasers Light Ophthalmol 1991;4:103–110.
24. Obana A, Miki T. Dye laser photocoagulation through experimental induced retinal hemorrhage. Lasers Surg Med 1991;11:152–157.
25. Nakajima S, Sakata I, Takemura T, et al. Tumor-localizing and photosensitization of photochlorin ATX-S10. In: Spinelli S, Fante D, Marchesini R, editors. Photodynamic therapy and biomedical lasers. Amsterdam: Elsevier; 1992. p 531–534.
26. Gohto Y, Obana A, Kaneda K, Miki T. Photodynamic effect of a new photosensitizer ATX-S10 on corneal neovascularization. Exp Eye Res (in press).
27. Tobe T, Takahashi K, Ohkuma H, Uyama M. Experimental choroidal neovascularization in the rat. J Jpn Ophthalmol Soc 1995;99:784–791.
28. Gohto Y, Obana A, Miki T, Kaneda K, Sakata I, Nakajima S. Mechanism of photodynamic effects of a photosensitizer (ATX-S10) on corneal neovascularization. ARVO abstracts. Invest Ophthalmol Vis Sci 1996;37: S547.
29. Gohto Y, Obana A, Miki T, Kaneda K, Nakajima S, Takemura T. Time course for the distribution of a photosensitizer (ATX-S10) to corneal neovascularization. ARVO abstracts. Invest Ophthalmol Vis Sci 1997;38:S513.
30. Kohno T, DeLaey JJ, Miki T. Detection of choroidal neovascularization in age-related macular degeneration using subtraction methods in indocyanine green angiography. Bull Soc Belg Ophthalmol 1995;259:81–88.
31. Gass JDM. Stereoscopic atlas of macular disease and treatment. 3rd ed. St Louis: CV Mosby; 1987. p 70–73.
32. Gomer CJ, Jester JV, Razum NJ, Szirth BC, Murphree AL. Photodynamic therapy of intraocular tumors: examination of hematoporphyrin derivative distribution and long-term damage in rabbit ocular tissue. Cancer Res 1985;45:3718–3725.
33. Haimovici R, Kramer M, Miller JW, et al. Localization of lipoprotein-delivered benzoporphyrin derivative in the rabbit eye. Curr Eye Res 1997;16:83–90.
34. Roberts WG, Berns MW. In vitro photosensitization 1. Cellular uptake and subcellular localization of mon-L-aspartyl chlorine e6, chloro-aluminum sulfonated phthalocyanine, and photofrin II. Lasers Surg Med 1989;9:90–101.